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## Hippo/YAP pathway for targeted therapy

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# Hippo/YAP pathway for targeted therapy

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**Abstract:** Malignant pleural mesothelioma (MPM) is molecularly characterized by loss of function or mutations in the neurofibromin 2 (NF2) and the cyclin-dependent kinase inhibitor 2 genes. NF2 activates a cascade of kinases, called Hippo pathway, which downregulates Yes associated protein (YAP) function as transcription co-activator for TEA domain transcription factors (TEAD). In the absence of functional NF2, the expression of genes essential for cell cycling such as survivin is increased. New therapeutic strategies aimed at interfering with YAP activity include inhibition of hedgehog pathway, which downregulates the YAP protein, verteporfin, which inhibits the assembly of a functional YAP-TEAD transcription factor, and interference with thrombin and lysophosphatidic acid (LPA) receptors downstream signalling, since upon agonist binding they activate YAP.

**Keywords:** Hippo pathway; Yes associated protein (YAP) oncogene; malignant pleural mesothelioma (MPM)

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## Introduction

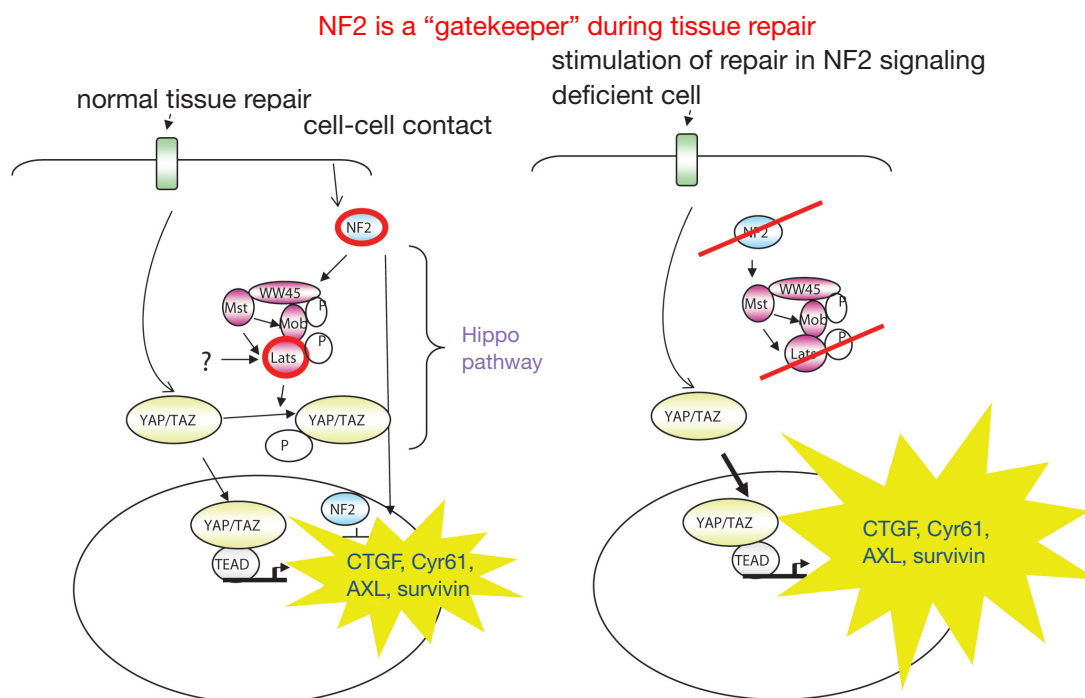
The Hippo signaling pathway controls organ size through the regulation of cell cycle, proliferation and apoptosis (1,2). It was discovered and linked for the first time to human cancer in 2002 (3). In this review we will briefly introduce this pathway, with an emphasis on components that are altered in human cancers, and then we will focus on its role in malignant pleural mesothelioma (MPM) and finally present potential implication for the therapy of MPM.

## The Hippo/Yes-associated protein (YAP) pathway in cancer

The mammalian components of Hippo pathway (*Figure 1*) include Serine/threonine kinase 3 and 4 (STK3 and 4, also called MST2 and 1, orthologs of *Drosophila* Hippo), SAV1, and serine/threonine kinase large tumor suppressor 1 and 2 (LATS1 and 2) (4). Neurofibromin 2 (NF2) (product of *NF2* gene), also called merlin, a member of the Ezrin ezrin/radixin/moesin protein family, promotes plasma membrane association of LATS which results in phosphorylation and activation of LATS1/2 by MST and other not yet known

kinases (5). Activation of LATS inhibits the transcriptional co-activator YAP and the co-activator with PDZ-binding motif (TAZ) through their phosphorylation. Indeed, phosphorylated YAP/TAZ cannot accumulate into the nucleus and this hinders their co-transcriptional activity. The dysfunction of Hippo pathway, which leads to increased YAP/TAZ activity with an underphosphorylated form in the nucleus (6), induces oncogenic transformation due to the activation of transcription factors including transcription enhancers activation domain (TEAD) family members (7). In mammals, there are four TEAD family members: TEAD1-4 which have a distinct but not mutually exclusive expression pattern (8). TEAD on its own is unable to induce gene expression and requires additional factors or co-activators for gene expression (8). Upon binding TEADs YAP/TAZ up-regulates the expression of several growth promoting factors, including secretory proteins connective tissue growth factor (CTGF) and Cyr61 (7), AXL receptor tyrosine kinase (9), c-myc and survivin (10,11).

Amplification of YAP-containing chromosome 11q22 amplicon is frequently observed in several human tumors. High levels of YAP are observed in human liver tumors and YAP is a key driver of hepatocellular carcinoma



**Figure 1** In normal tissue repair NF2 activates the Hippo pathway, an essential regulator of cell proliferation. Key components of the Hippo pathway include two kinases: Mst and Lats. The sequential activation of these kinases leads to phosphorylation of the transcription factor YAP. When Hippo signaling is attenuated, e.g., in NF2-deficient cancer cells, YAP phosphorylation is reduced, resulting in its nuclear localization and regulation of target genes such as CTGF, Cyr61, AXL and survivin. Survivin is also controlled by nuclear NF2.

tumorigenesis (12,13). High YAP levels are also seen in about 15% of ovarian cancers where it has been correlated with poor patient prognosis (14). Similarly, a correlation between high YAP expression and poor prognosis has been identified in non-small cell lung cancer and esophageal squamous cell carcinoma (15,16). YAP overexpression is also seen in medulloblastoma (17) intracranial ependymoma (18) and oral squamous cell carcinoma (19). Also, YAP plays a role in human colorectal cancer progression (20). Mutations in Mst1, Mst2, LATS1 and LATS2 are not common but epigenetic silencing of these genes has been reported (21-23).

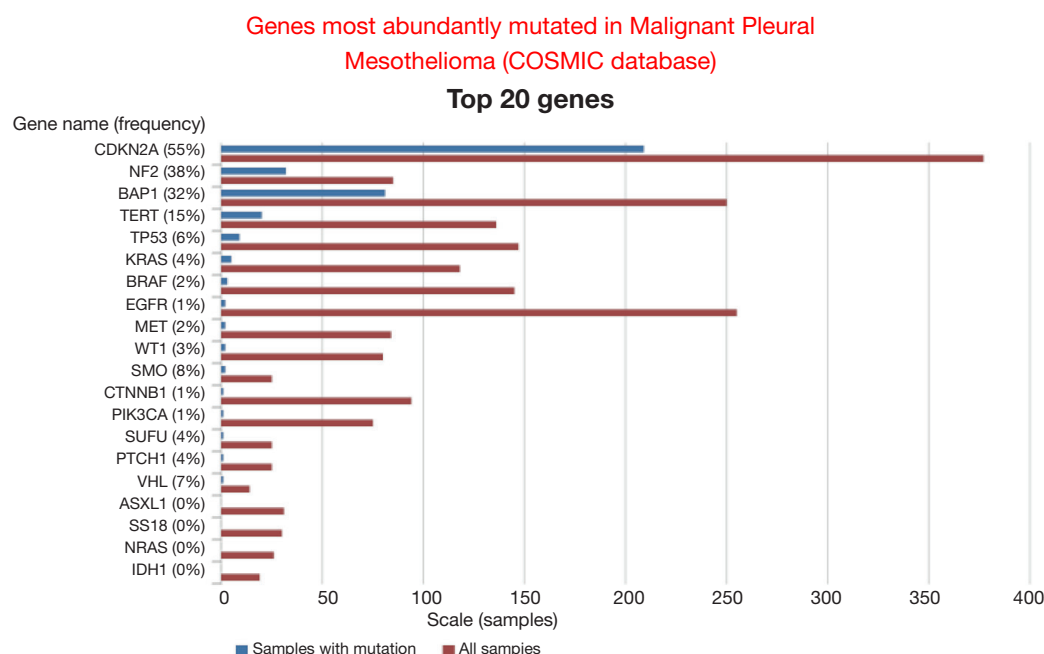
Hippo pathway activity may also be altered through crosstalk with other signalling pathways, which harbour oncogenic alteration [reviewed in (24)]. The Hippo pathway has been implicated in cell contact inhibition, as YAP/TAZ display a dramatic cell density dependent subcellular localization and phosphorylation (25). In addition, mechanic stress has also been shown to modulate YAP/TAZ activity (26). Recent studies have shown that YAP activity is regulated by G-protein-coupled receptor signaling (27,28). In this context it is of particular interest the activation of YAP/TAZ by thrombin and protease-

activated receptor PAR1 (29), since thrombin is generated at sites of tissue injury to promote wound healing. In addition inhibition of mevalonate pathway with simvastatin decreases nuclear YAP (30).

The interaction with sonic hedgehog stem signalling pathway in mesothelioma will be detailed below.

### The Hippo/YAP pathway in MPM

MPM is an aggressive human malignancy (31). MPM is mostly associated with asbestos exposure and the latency period after initial exposure is typically longer than 30 years (32). MPM is a rare disease with a 15-year cumulative frequency during 1994-2008 in the 56 countries reporting MPM to be 174,300 (33); however the real incidence of MPM is unknown, since there are countries in which MPM mortality is not reported, including asbestos-producing countries such as Russia, Kazakhstan, China and India (33). MPM mortality rates are estimated to increase by 5-10% per year in most industrialized countries until about 2020 (34). Despite treatment with chemotherapy, radiation therapy or surgery, the disease carries a poor prognosis. The median survival time



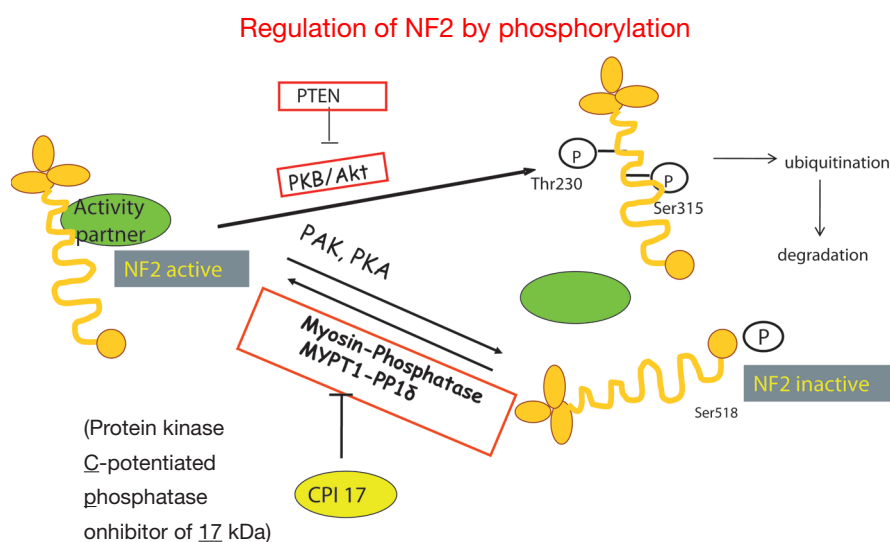
**Figure 2** Data mining of version 67 of the catalogue of somatic mutations in cancer (COSMIC, <http://www.sanger.ac.uk/cosmic>), reveals that the genes that are mostly mutated in malignant pleural mesothelioma are *cyclin-dependent kinase activator inhibitor (CDKN2A)*, *neurofibromatosis type 2 (NF2)* and *BRCA-associated protein 1 (BAP1)*.

of patients after diagnosis is only 7-12 months (35).

The mechanism of development of MPM after exposure to asbestos fibres is not well understood. Few hypotheses can be proposed based on experimental data and observation of clinical samples, some of which has been detailed in several reviews (36-42). Chronic tissue repair activates stem cell signalling pathways to regenerate the tissue but, because of persistent system stimulation, oncogenic events occur leading to the formation of a tumor (43). Cells that are stimulated to proliferate upon asbestos fibres exposure may be undifferentiated precursor cells, as it has been shown for mesothelial regeneration after injury (44). Undifferentiated precursor cells have been recently described in normal mesothelial primary cultures (45). The activation of stem cell signalling would normally be kept under control, but, because of persistent system stimulation, oncogenic events occur leading to the formation of a tumor (43). In line with such hypothesis one would expect the oncogenic events to occur within the components responsible for homeostasis in tissue repair and in control of stem cell signalling. Activated stem cell signalling has already been suggested in MPM by the presence of an 11-gene signature, correlated with a stem-cell-like expression profile, which is associated with a poor prognosis in patients with MPM (46). Knowledge

about common alterations observed in MPM, which are detailed below, confirms that alteration in NF2 signalling, which is responsible for homeostasis in tissue repair and in the control of stem cell signalling, is the ideal target for an oncogenic event to occur during the development of MPM. This hypothesis is supported by the observation that Hippo pathway restricts the oncogenic potential of intestinal regeneration program induced after injury by dextran sodium sulfate (47).

Data mining of version 68 of the catalogue of somatic mutations in cancer (COSMIC, <http://www.sanger.ac.uk/cosmic>) (48) reveals that the genes that are mostly mutated in MPM (Figure 2) are *cyclin-dependent kinase activator inhibitor (CDKN2A)*, *neurofibromatosis type 2 (NF2)* and *BRCA-associated protein 1 (BAP1)*. Although the total number of samples (<500, status February 2014) screened is too low to confidently predict mutation frequencies this estimate is nevertheless useful to infer a general MPM profile. *CDKN2A* and *NF2* are the two most abundantly mutated genes in MPM. Indeed, MPM lack expression of both *CDKN2A* encoded proteins p16 and ARF (49,50) due to gene deletion (51-53) or methylation (54-56). Mutations in *NF2* gene have been found in about 40% of mesothelioma (57-59). In MPM tumors with no detectable genetic



**Figure 3** The function of tumor suppressor NF2 is inactivated by genetic alterations or it is controlled by phosphorylation, which depending on the phosphorylated amino acid, leads to functional inactivation or AKT-dependent degradation. NF2 phosphorylation on Ser518 is favored when levels of protein kinase C-potentiated phosphatase inhibitor of 17 kDa (CPI-17), which inhibits the phosphatase reactivating NF2, are high. On the other hand PTEN deletion results in increased AKT activity and NF2 degradation.

alterations of NF2, its activity is downregulated. Indeed, the activity of NF2 is controlled by phosphorylation (Figure 3), which depending on the phosphorylated amino acid, leads to functional inactivation (60,61) or AKT-dependent degradation (62). Experimental animal models indicate that disruption of the NF2 signalling pathway, together with a deficiency in *CDKN2A*, is essential for mesothelioma development (63-65). NF2 is an upstream regulator of the Hippo signaling cascade, which is conserved from *Drosophila* to mammals (10). The NF2/Hippo connection is supported by mouse genetics, wherein heterozygosity of *Yap* greatly suppresses the *Nf2*-deficient phenotype (66).

It has been proposed that genes which are inactivated in a given tumor type and directly regulate tumor growth by either inhibiting growth or promoting death are “gatekeeper” genes (67). According to the data mentioned above, NF2 does correspond to this definition and should be considered a “gatekeeper” in mesothelioma. Indeed, NF2 mediates contact-dependent inhibition of proliferation by both sensing cell-cell contact and intercepting mitogenic signalling initiated at the plasma membrane (68). In cultured mammalian cells, NF2 inhibits internalization, effector complexing and downstream signalling of activated EGFR upon cell-cell contact. This is consistent with the idea that NF2/merlin normally sequesters EGFR into non-signalling plasma membrane compartments (69). In addition, NF2

is required for the assembly, but not the maintenance, of apico-lateral junctional complexes (70) which means that NF2 loss will be most important when it occurs in dividing cells, for example during tissue repair. Cells that cannot form apico-lateral junctional complexes will be unable to form a well-organized tissue and will be resistant to contact-dependent growth arrest. A role of NF2 in tissue repair is further supported by the observation that the active form of NF2 suppresses tumorigenesis by migrating into the nucleus where it inhibits the E3 ubiquitin ligase CRL4 and through that controls a subset of Hippo pathway target genes (71). This recent observation is consistent with previous evidence of NF2 signaling-dependent activation of the Hippo pathway (72).

Data from the group of Sekido (73) and the group of Ladanyi (74) indicate that NF2/Hippo signalling is disrupted in most MPM. It is important to note that downstream of such a disrupted signalling, TEAD1/YAP1 are activated and YAP/TEAD1 are necessary, but not sufficient (75), for the overexpression of mesothelin. Mesothelin is expressed in normal mesothelium (76) and is a marker of epithelioid mesothelioma (77).

YAP is constitutively active in more than 70% of primary MPM (73), it has been originally described in an inducible transgenic model to be involved in organ size control paralleled by a 30-fold increase in survivin expression (10). A

recent study has showed that it controls survivin expression in MPM (78). We have observed that Hedgehog signaling is activated in MPM, consistent with the re-activation of a signalling known to be essential during embryonic mesothelium development (79). Treatment with an inhibitor of Hedgehog pathway (HhAntag) of mesothelioma cells, grown in cell culture conditions favoring stemness, was arresting cells growth and this was accompanied by decreased levels of survivin (80). Survivin is not described as a direct target downstream Hedgehog pathway. Hence, we sought for other transcription activators known to be expressed in MPM and to regulate survivin expression and the most obvious was YAP. We confirmed nuclear expression of YAP in MPM and observed that HhAntag reduced YAP protein levels. Transient transfection of a constitutively active YAP (81) rescued HhAntag-dependent survivin decrease, confirming the interaction between Hedgehog and YAP signaling. Tumor bearing mice were randomized in two groups receiving either solvent or HhAntag. The *in vivo* HhAntag treatment dosage (38 mg/kg bw, administered twice daily by oral gavage, 5 d/week) was chosen based on therapeutic range reported in the literature (82). HhAntag led to a significant 35% decrease of the tumor volume after the two weeks of treatment. At the end of dosing regimen, animals were euthanized in order to collect tumor tissue for RNA extraction and immunohistochemical analysis. A different expression profile indicating changes in both tumor and stromal tissue were obtained in the two groups. The effect of HhAntag on tumor volumes was also accompanied by a significant 43% decrease in Ki-67 labelling index. Furthermore, consistent with *in vitro* experiments, we observed a significant 32% decrease in nuclear YAP immunostaining in HhAntag treated tumors. The observation that HhAntag decreases YAP protein is consistent with the role of Hedgehog signaling in maintaining YAP protein stability (17).

More recently, the modulation of YAP by the AJUBA family has been investigated in MPM (83). The mammalian Ajuba family comprises three proteins AJUBA, LIMD1, and WTIP characterized by a so-called, LIM domain. The LIM domain defines a cysteine-rich double zinc finger initially identified in three developmentally important transcription factors, *Caenorhabditis elegans* Lin-11, rat Isl-1, and *C. elegans* mec-3, from which the acronym LIM is derived (84). Although in *Drosophila* the unique AJUBA family member ortholog activates YAP by binding LATS (85), in MPM the three different LIM family members seem not to have all

the same YAP-activating properties (83).

### Implications for therapy

Under the hypothesis that disruption of NF2 function acts as “driver” in MPM, therapeutic intervention on genes that are normally kept under control by NF2 and the Hippo pathway such as, e.g., survivin, would be a reasonable approach. However, this might not be easy to implement. In the context of MPM harboring mutated NF2/Hippo pathway, cancer cells may be addicted to the activity of YAP. Liu-Chittenden *et al.* (86) screened a Johns Hopkins Drug Library, a collection of >3,300 drugs, for compounds that could inhibit the transcriptional activity of YAP *in vitro*. Three compounds related to porphyrin were identified with this assay. One of these, verteporfin, is in clinical use as a photosensitizer in photocoagulation therapy for macular degeneration. Verteporfin was moderately effective at blocking mouse *Yap1*-overexpression- or loss of *Nf2*-driven hepatic tumorigenesis. These data suggest the application of these compounds as anticancer therapies independently of their photosensitizing roles.

Downstream G-coupled receptor signalling we mentioned above YAP activation via thrombin/PAR1 (29) and this activation could be relevant in MPM that depend on PAR1 for growth (87). In these cases one potential option for therapy might be PAR-1 antagonists pepducins such as P1pal-12 (88) which is a cell-penetrating peptide derived from the third intracellular loop of PAR-1. Once inserted into the plasma membrane it is delivered to the PAR-1 intracellular surface, thereby interfering with the receptor/G-protein interaction. Lysophosphatidic acid (LPA) stimulates YAP activity (27) and has also been described to stimulate MPM growth (89), offering another opportunity for intervention.

Simvastatin decreases nuclear YAP and induces growth arrest by interfering with protein geranylgeranylation (30). The same mechanism may participate to lovastatin-mediated protective effect against cisplatin in proliferating normal mesothelial cells (90).

Other druggable targets, which might be relevant in other cancer types, have been summarized in two recent reviews (91,92).

In MPM the strategy may depend on upstream signalling: activated stem signalling, thrombin/PAR1 and LPA/LPA receptor are all potentially interesting targets to inhibit in view of interfering with YAP activation. Nevertheless, for



the time being, only for Hedgehog stem signalling a direct link with YAP activation has been demonstrated (80).

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